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## Effect of Temperature on Growth and Biochemical Composition (Sterols, $\alpha$ -tocopherol, Carotenoids, Fatty Acid Profiles) of the Microalga, *Isochrysis galbana*

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### Abstract

The microalga *Isochrysis galbana* Parke was grown in 100-l polyethylene bags filled with sterilized seawater enriched with Wallerstein and Miquel nutrient medium (3:1) at two temperatures (18°C and 26°C), 2.5% salinity, and continuous illumination. Samples of microalgae were examined during the exponential growth, stationary growth, and decay phases (days 18, 35, and 58, respectively). Stigmasterol constituted the main sterol group in all phases in both treatments, except during the exponential growth phase at 18°C. The highest concentration of  $\alpha$ -tocopherol was 5.22 mg/100 g DW, reached in the decay phase of algae raised in 18°C. Likewise, the highest total carotenoid (4.14 mg/g DW) and chlorophyll (2.87 mg/g DW) levels were obtained in algae raised in 18°C, during the exponential phase; the main pigments were chlorophyll *a*, *c1*, *c2*, and fucoxanthin. At 18°C, polyunsaturated fatty acids (PUFA) were the predominant fatty acid group, reaching 37.93% of the total fatty acids in the stationary phase. At 26°C, saturated fatty acids (SAF) were the predominant group of fatty acids. The present study suggests that the nutritional value (gross biochemical composition and fatty acid profile) of *I. galbana* Parke is, to a large extent, determined by the temperature at which it is grown and the harvest time.

### Introduction

Microalgal culture is a key procedure in fish hatcheries, but this activity is far from optimized and several problems remain to be solved. In particular, the amount of good quality microalgae available in hatcheries is limited

and several species which were previously used in commercial hatcheries have been discarded due to their poor nutritional value (Durmaz, 2007). Some problems in aquacul-

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ture are related to breeding different fish species in different seasons. Farms cannot always optimize algae production in accordance with the breeding of each species. In Turkey, an aquaculture farm can breed sea bream or sea bass in autumn but cannot always breed other fish species that spawn in warmer seasons. One of the reasons for this may be a decrease in quality of live feed resulting from changes in temperature of the algae culture medium.

The golden-brown flagellate *Isochrysis galbana* is a rich source of polyunsaturated fatty acids (PUFA), mainly eicosapentaenoic acid, EPA, 20:5n-3 (Molina-Grima et al., 1994; Fidalgo et al., 1998), making it a useful feed for aquatic animals (Sanchez et al., 2000). Its richness in PUFA makes it promising as an animal and/or human nutraceutical food (Otles and Pire, 2001). *Isochrysis galbana* synthesizes important bioactive metabolites such as sterols (Volkman et al., 1981), tocopherols (Fabregas and Herrero, 1990), carotenoids (Flynn et al., 1993), and pharmaceuticals (Fabregas et al., 1985). Thus, it is commonly cultivated in fish hatcheries as a feed for rotifers, creating a "green-water effect" in fish larvae tanks. In hatcheries, the marine microalgae *I. galbana* are often cultivated in transparent polyethylene bags or fiberglass cylinders under artificial light (Richmond, 1986; Boussiba et al., 1988).

The chemical composition of microalgae is influenced by environmental conditions such as temperature and growth phase (Fabregas et al., 1985; Kaplan et al., 1986; Richmond, 1986; Qiang and Richmond, 1994; Renaud et al., 1995), growth rate, irradiance (Molina-Grima et al., 1994), nutrient concentration (Fabregas et al., 1985), and solar cycle (Molina-Grima et al., 1995). However, the response to growth temperature varies from species to species, with no overall consistent relationship between temperature and fatty acid unsaturation (Thompson et al., 1992; Renaud et al., 1995). Renaud et al. (1995) concluded that the optimum temperature range for a reasonable growth rate, maximum lipid production, and high levels of essential fatty acids is 20-30°C for *Isochrysis* sp.

However, since the biochemical composition and the growth rate of *I. galbana* are subject to temperature changes, it is necessary to culture a suitable strain of *I. galbana* algae in each season.

The aim of this study was to compare production of *I. galbana* Parke at two temperatures: 18°C and 26°C.

### Materials and Methods

The marine microalgae *I. galbana* (Haptophyceae) were obtained from the Mary Park collection and maintained at the Department of Aquaculture in IPIMAR (Lisboa, Portugal). The study was performed in the IPIMAR aquaculture laboratory. Pigments were analyzed at the *Instituto Nacional de Engenharia e Tecnologia Industrial* (Lisboa, Portugal).

The microalgae were grown in 100-l polyethylene bags (165 x 25 cm) in a batch-culture system in sterilized seawater enriched with Wallerstein and Miquel medium (3:1). Salinity was 2.5 g/l. Triplicate cultures were kept at a constant room temperature of 18±1°C or 26±1°C and continuous illumination from fluorescent lamps (Philips TLM 40W/54RS). Photon flux density at the surface of the bags was 196 µmol m<sup>-2</sup>/s (Li-Core 195).

The number of cells was measured with an electronic particle counter (Coulter EPICS XL, Beckman Company, Miami, FL). Instantaneous growth rates (µ; division/day) were calculated using the equation:  $\mu = (\ln N_t - \ln N_0) / (t - t_0)$ , where  $N_t$  is the number of cells at time  $t$  and  $N_0$  is the number of cells at time  $t_0$ .

Samples of microalgae were collected during the exponential growth, stationary growth, and decay phases (days 18, 35, and 58, respectively) by flocculation with FeCl<sub>3</sub>, followed by centrifugation as reported by Batista and Martins (1991). The resultant pellet was freeze-dried for further analysis, as follows.

Sterols were extracted by a method adapted from Bandarra et al. (2003). Extraction began with a saponification step by mixing 0.25 g of the freeze-dried material with 2.5 ml of 2M methanolic KOH solution and 100 µl of 0.5% α-cholesterol in chloroform.

Trimethylsilylethers were analyzed in a gas chromatograph (Hewlett Packard HP 5890 A Series, Albertville, MN) equipped with a flame ionization detector at 300°C with manual injection in a split injector (100:1 split ratio) at 285°C. Separation was carried out with helium as the carrier gas at a flow of 1 ml/min in a capillary column HP-5 (30 m x 0.25 mm i.d. x 0.25 µm film thickness; Albertville, MN) programmed to a constant temperature of 280°C.

The vitamin  $\alpha$ -tocopherol was extracted immediately after freeze-drying following a method adapted from Chen et al. (1998). The organic phases were pooled and a 20-µl aliquot was injected in a HPLC (JASCO model 980, Japan) equipped with an automatic injector (JASCO Model AS-950-10, Japan) and a fluorescent detector (JASCO Model FP-1520,  $\lambda_{exc}$  = 290 nm and  $\lambda_{em}$  = 300 nm). Separation was carried out in a Lichrosorb Si 60-5 column (250 mm x 3 mm i.d.; Chrompack, USA) protected by a silica pre-column S2-SS (10 mm x 2 mm i.d.; Chrompack, USA). The mobile phase was a mixture of n-hexane and isopropanol (99.3:0.7 v/v) degassed in a Gastor Model GT-104 System (Japan) and eluted at a constant flow of 1 ml/min. Data was recorded and analyzed using Borwin chromatographic software (version 1.21, France).

Total carotenoid content was determined spectrophotometrically after extraction with acetone (Choubert and Storebakken, 1989). Carotenoids are expressed using extinction coefficients ( $\epsilon_{cm}^{0.01\%}$ ) of 2150 for algal pigments at their absorption maximum in acetone (Gouveia et al., 1997). Individual pigments were detected by HPLC (Perkin Elmer), reversed-phase, with a  $\mu$ -Bondapak C18 column and a detector UV/VIS (Waters 481,  $\lambda$ =460 nm), with methanol:acetonitrile:water (65:35:2) as the eluent. Methanol and acetonitrile were HPLC-grade reagents, used with no further purification other than filtration and degassing. The pigments were eluted over 20 min at a flow of 1 ml/min.

Fatty acid methyl esters were prepared according to Lepage and Roy (1986) modified by Cohen et al. (1988), analyzed in a gas chromatograph (Varian Star 3400 Cx, Walnut Creek, CA) equipped with an auto-sampler

and fitted with a flame ionization detector at 250°C, and separated in a 30-m polyethylene glycol capillary column DB-WAX (0.25 mm i.d. x 0.25 µm film thickness; J&W Scientific, Folsom, CA). The column was subjected to a temperature program of 180°C for 5 min, 4°C for 10 min, and 220°C for 25 min. The temperature of the injector (split ratio 100:1) was kept constant at 250°C during the 40 min analysis.

Unless otherwise stated, all reagents were from Merck (Darmstadt, Germany) and standards from Sigma-Aldrich (St. Louis, USA).

The Kolmogorov-Smirnov test was used to verify the normality and homogeneity of variances. Data were analyzed using ANOVA. When data did not meet the assumptions of ANOVA, the Kruskal-Wallis test (a nonparametric ANOVA equivalent) was performed. The sources of significant differences were determined using the Tukey test (SPSS 9.0 software; Zar, 1999).

## Results

Cell density at 18°C increased rapidly to  $4.97 \times 10^6$  cells/ml by day 11 without any apparent lag during the exponential phase; the maximum specific growth rate was 0.20 division/day (Fig. 1). At 26°C, there was a lag between days 0 and 7, then a gradual increase in density; the maximum specific growth rate, recorded at day 11, was 0.12 division/day.

Four sterols were detected: campesterol, stigmasterol, and  $\beta$ -sitosterol, and an unidentified monomethylsterol (Fig. 2). At 18°C,  $\beta$ -sitosterol dominated during the exponential growth phase (162.39 mg/100 g DW) but stigmasterol was the main sterol in the stationary (137.60 mg/100 g DW) and decay (164.40 mg/100 g DW) phases. Stigmasterol dominated all phases at 26°C (15.50-41.20 mg/100 g DW).

There was a significant difference ( $p < 0.05$ ) between  $\alpha$ -tocopherol in the exponential phase (2.25 mg/100 g DW) and in the decay phase (5.22 mg/100 g DW) at 18°C (Fig. 3). Tocopherol levels at 26°C were much lower than at 18°C, decreasing from 0.26 mg/100 g DW in the exponential phase to 0.17

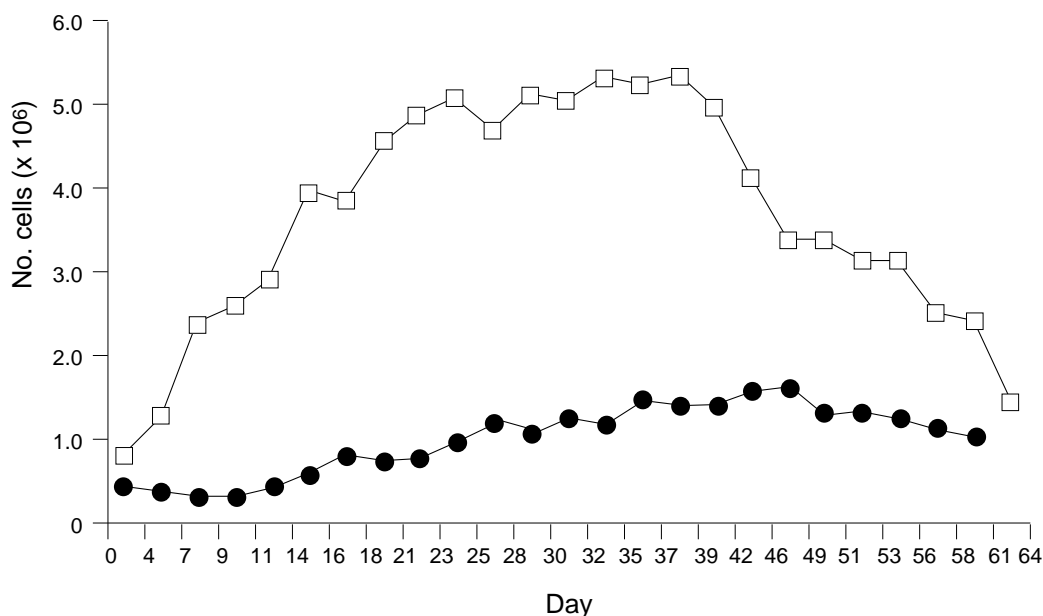


Fig. 1. Number of *Isochrysis galbana* cells at 18°C (□) and 26°C (●).

mg/100 g DW in the decay phase. Total carotenoids and chlorophyll levels decreased from the exponential phase to the decay phase in both temperatures. The main pigments detected in all phases were chlorophylls a, c1, and fucoxanthin.

The proportion of total polyunsaturated fatty acids (PUFA) was considerably higher while saturated fatty acids (SFA) were considerably lower in all stages in cultures grown at 18°C than in cultures grown at 26°C (Table 1). PUFA was the dominant group in all phases at 18°C while SFA was the dominant group at 26°C. At 18°C, EPA (20:5n-3) was the major PUFA, accounting for over 20% of the total fatty acids in the each stage. In the 18°C treatment, the highest PUFA content was in stationary phase, 37.9%, while in the same phase in the 26°C treatment, it was only 14.7%. Palmitoleic acid (16:1n-7) was the main mono-unsaturated fatty acid (MUFA) at both temperatures. The highest MUFA levels at 18°C and 26°C were similar ( $p>0.05$ ) but detected in different phases.

### Discussion

In this study, *I. galbana* Parke was cultured in 100-l plastic bags, similar to commercial aquaculture farms, but at a relatively higher temperature (26°C). As a result, both growth rate and biochemical composition decreased in comparison with *I. galbana* cultured at 18°C. Results show that a compromise between nutritional properties and growth kinetics of *I. galbana* can be achieved at 18°C, allowing for high specific growth and biomass productivity values. This finding is in agreement with Fabregas et al. (1985, 1986) whose optimum temperature was 15–22°C but contrasts with previously suggested optimum temperatures of 25°C (Kaplan et al., 1986) and 27±1°C (Qiang and Richmond, 1994).

In our study, stigmasterol and  $\beta$ -sitosterol were the main sterols identified in *I. galbana*, as reported by Bandarra et al. (2003). However, Bandarra et al. (2003) reported that the highest level was in the decay phase,

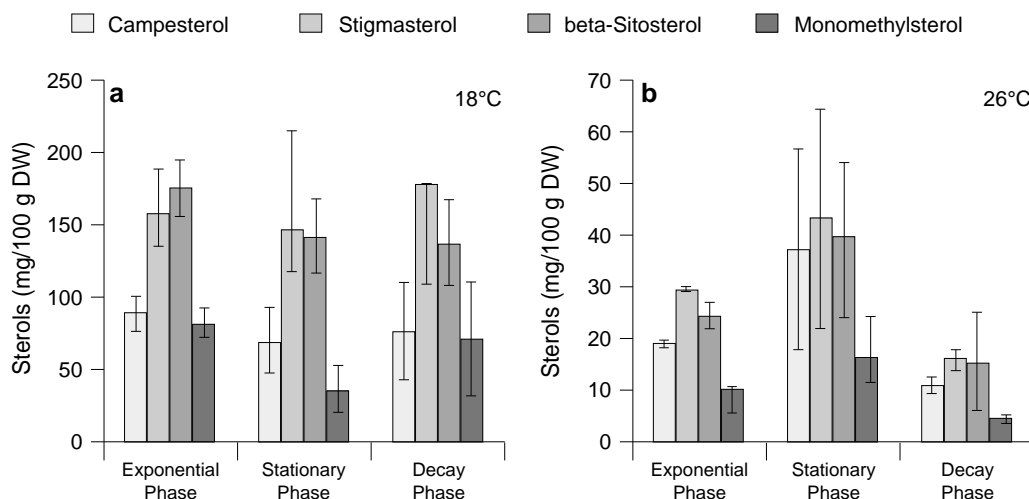


Fig. 2. Sterols in samples of *Isochrysis galbana* grown at (a) 18°C and (b) 26°C; samples were taken on days 18 (exponential growth phase), 35 (stationary growth phase), and 58 (decay phase).

whereas in our study the lowest level was detected in the decay phase. Epibrassicasterol or its isomer brassicasterol was identified as the predominant sterol by Patterson et al. (1994), Volkman et al. (1981), and Goad et al. (1982). The variable sterol pattern can be attributed to different *Isochrysis* strains (Patterson et al., 1994), suggesting an important subject for future taxonomic study.

The increase of  $\alpha$ -tocopherol during the growth phases at 18°C was also observed by Bandarra et al. (2003), however, in the study of Bandarra et al. (2003),  $\alpha$ -tocopherol reached a maximum of 8.85 mg/100 g DW. This difference can be due to the high instability of this compound and the important role of this antioxidant in oxidation reactions during ageing. Fabregas and Herrero (1990) registered 0.58 mg/100 g DW during the decay phase of this microalgae, which is considerably low but can be due to loss during oven-drying at 60°C for 24–36 h. At 26°C,  $\alpha$ -tocopherol production was very low and decreased during the growth phases. A similar decrease of carotenoids and chlorophylls in the decay phase was reported by other authors and can be related to the lower photosynthetic activity of the species at the end of growth (Fidalgo et al., 1998).

At 18°C, EPA accounted for 22.3% of the fatty acids in the stationary phase, in agreement with Bandarra et al. (2003), Fidalgo et al. (1998), and Medina et al. (1995). C14:0 and 16:0 were the main SFA, in agreement with Volkman et al. (1981). The main MUFA was 16:1n-7, accounting for up to 16.0% (at 26°C, stationary phase). A lower percentage of this FA (0.7% in the exponential phase) was found by Volkman et al. (1981). Total lipid, EPA, and docosahexaenoic (DHA) levels are inversely related to temperature, as observed for *Isochrysis* and other microalgae (Tasselli and Doimi, 1990).

Our findings indicate that both growth and biochemical composition (i.e., sterols,  $\alpha$ -tocopherol, carotenoids, and fatty acids) of the microalgae *I. galbana* Parke can be maximized by growing them at 18°C. The optimum growth temperature for the *I. galbana* clone T-ISO, *Isochrysis* sp., CCMP 463 and 1324 strains, is relatively high, i.e., 26–30°C, while for *I. galbana* Parke it is 18–20°C. Because the biochemical composition and growth rate of *I. galbana* are subject to temperature changes, it is necessary to culture a suitable strain of *I. galbana* algae in each season. By doing so, the success of breeding alternative fish species in mariculture operations will be increased.

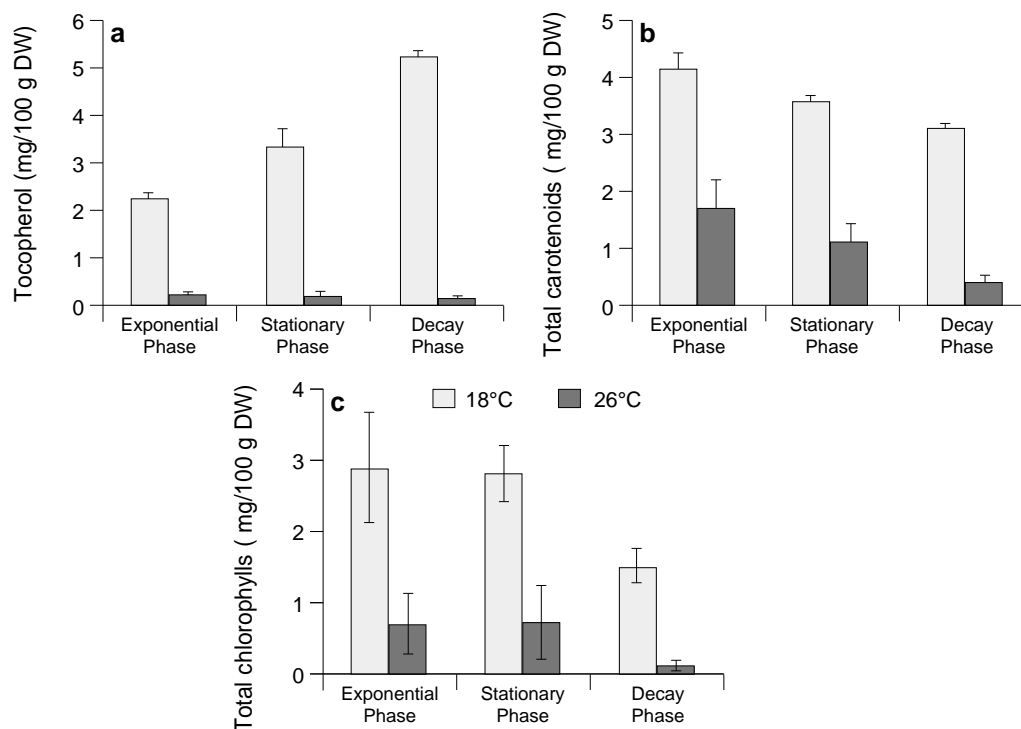


Fig. 3. Levels of (a) tocopherol, (b) total carotenoids, and (c) total chlorophylls in samples of *Isochrysis galbana* grown at 18°C and 26°C taken on days 18 (exponential growth phase), 35 (stationary growth phase), and 58 (decay phase).

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Table 1. Fatty acid profiles of *Isochrysis galbana* microalgae cultures grown in 18°C or 26°C and harvested in the exponential growth (day 18), stationary growth (day 35), and decay (day 58) phases.

Fatty acid (%)	18°C			26°C		
	Day 18	Day 35	Day 58	Day 18	Day 35	Day 58
18:4n-3	5.24±0.008	5.15±0.13	4.40±0.10	0.78±0.55	1.49±0.38	1.24±0.54
20:5n-3	20.18±0.36	22.30±0.54	20.48±1.70	5.86±2.74	8.04±2.37	6.54±1.75
22:6n-3	5.26±0.18	5.02±0.28	4.35±0.83	1.65±1.11	1.86±0.52	2.81±0.68
Other PUFA <sup>1</sup>	5.13±0.09	5.46±0.13	6.13±0.51	6.13±2.87	3.35±0.99	3.55±0.95
<b>Total PUFA</b>	<b>35.81±0.72</b>	<b>37.93±0.47</b>	<b>35.36±3.24</b>	<b>14.42±4.24</b>	<b>14.74±3.73</b>	<b>14.14±1.92</b>
14:0	18.97±0.18	13.89±0.25	13.76±0.90	15.32±0.85	19.27±1.47	18.57±1.82
16:0	13.23±0.17	13.85±0.39	13.75±0.82	22.26±1.50	22.57±2.02	24.54±3.76
Other SFA <sup>2</sup>	2.30±0.02	2.58±0.05	2.34±0.15	5.27±0.01	3.03±0.23	3.75±0.37
<b>Total SFA</b>	<b>34.50±0.28</b>	<b>30.32±0.40</b>	<b>29.85±1.53</b>	<b>42.85±2.63</b>	<b>44.87±3.78</b>	<b>46.86±5.48</b>
16:1n-7	14.63±0.20	13.95±0.45	15.80±1.22	7.71±3.89	16.05±0.52	11.65±4.69
Other MUFA <sup>3</sup>	2.95±0.04	4.14±0.13	5.17±0.40	5.48±2.76	5.13±0.17	3.35±1.35
<b>Total MUFA</b>	<b>17.58±0.18</b>	<b>18.09±0.48</b>	<b>20.97±1.69</b>	<b>13.19±3.52</b>	<b>21.18±0.84</b>	<b>15.00±5.74</b>

<sup>1</sup> Includes 16:4n-3, 18:2n-6, 18:3n-3, 18:4n-3, 20:4n-3, 20:4n-6, 20:4n-3, 22:5n-6, 22:5n-3

<sup>2</sup> Includes 12:0, 13:0, 14:0 isobr, 15:0, 16:0 iso, 16:0 anteiso, phytanic acid, 18:0, 20:0, 22:0

<sup>3</sup> Includes 17:1, 18:1n-7, 18:1n-9, 20:1n-9, 20:1n-7, 22:1n-11, 22:1n-9



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